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Short Communication

Purification and characterization of a new β-glucosidase from *Penicillium piceum* and its application in enzymatic degradation of delignified corn stover

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HIGHLIGHTS

- A new β-glucosidase (PpCel3B) was firstly isolated and characterized from cellulytic fungi.
- PpCel3B had a very high transglycosylation activity to produce disaccharide derivatives.
- PpCel3B, with new enzymatic activity against xylotriose, could form hemicellulase inducers.
- PpCel3B play an important role in forming inducers for cellulase and hemicellulase synthesis.
- Supplementing low doses of PpCel3B could enhance different cellulase efficiency by 15–35%.

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ABSTRACT

A new β-glucosidase (Cel3B) was first isolated from cellulytic fungi, designated as PpCel3B. Although PpCel3B was classified to GH family 3 based on the homology sequence, PpCel3B had different biological functions in cellulose degradation and signaling molecules production. PpCel3B was constitutive and could form multiple soluble lignocellulose inducers for cellulase and hemicellulase synthesis via high tranglycosylation activity and new enzymatic activity. Moreover, PpCel3B showed apparent synergism with cellulases by removing several inhibitors. Supplementing low doses of PpCel3B (52 μg/g substrate) increased saccharification efficiency of cellulase produced by *Trichoderma reesei* and *Penicillium piceum* by 15% and 35%, respectively on delignified corn stover. PpCel3B had important application in boosting cellulase yield and efficiency.

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1. Introduction

Lignocellulosic biomass is the only foreseeable sustainable source of fuels and materials available to humanity (Lynd et al., 2002). The conversion of lignocellulosic biomass needs a disruptive pretreatment and enzyme-catalyzed hydrolysis of cellulose and hemicellulose to fermentable sugars (Berlin et al., 2006). The combined action of the CBHs and EGs on cellulose generates mainly the short soluble oligosaccharides cellobiose, cellotriose and cellotetraose, which are cleaved to glucose monomers by the BGs (Foreman et al., 2003; Takashima et al., 1999). β-Glucosidase played a crucial role in large scale saccharification of cellulose by removing cellobiose that inhibits the cellobiohydrolase and endoglucanases (Medve et al., 1997; Singhania et al., 2013). Numerous studies have shown that cellulose hydrolysis is improved using cellulases from *Trichoderma reesei* supplemented with extra β-glucosidase activity (Tengborg et al., 2001; Tu et al., 2006).

β-Glucosidase is one among the earlier discovered and widely studied enzyme due to its universal distribution and well defined wide variety of substrate and simple nature of enzyme assay (Singhania et al., 2013). Recently, with the greater availability of fungal genome sequence data, evidence for the existence of multiple β-glucosidase genes in other species has emerged including two characterized β-glucosidases (BGL1/Cel1A and BGLII/Cel3A) and five predicted β-glucosidases (Cel1B, Cel3B, Cel3C, Cel3D, Cel3E) (Foreman et al., 2003). Cel3A and Cel1A were the major extracellular and intracellular β-glucosidase respectively. Other five β-glucosidases (Cel1B, Cel3B, Cel3C, Cel3D and Cel3B) was unknown so far. GH3 enzymes may have arisen from an ancestor, the evolution provided different biological functions for the identity family, with similar or same amino acid sequences (Collins et al., 2007). The speculative functional annotation of Cel3B transferred from Cel3A based on the similar sequence was not very accurate. The purification and characteristics of Cel3B have not been

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reported until to date. The real knowledge about the characteristics and functions of Cel3B was unknown.

This paper firstly elucidated the purification of Cel3B from *Penicillium piceum*. This new β-glucosidase was demonstrated new characterization and exact role in cellulose degradation and signaling molecules production. This results derived from experimental data could update and re-annotated real characteristic and functions of predicted enzymes, which could remove discrepancies between annotations in the different genome versions.

2. Methods

2.1. Microorganism and culture condition

P. piceum was a cellulase hyperproducing mutant stain obtained by chemical mutagenesis in our laboratory (unpublished data). Medium for cellulase production was (g/L): 2.7% microcrystalline cellulose, 3.3% corncob steep liquor, 0.5% $(NH4)_2SO_4$, 0.6% KH_2PO_4, 0.1% $MgSO_4$, 0.25% $CaCO_3$, 0.2% Tween-80. Spore suspension were inoculated into 50 mL of the liquid medium in a 300-mL conical flask, and grown at 28 °C with rotatory shaking at 180 rpm for 120 h.

2.2. Enzyme assay

β-Glucosidase activity was assayed with *p*-nitrophenyl β-D-glucopyranoside (*p*NPG) (Sigma, USA) as the substrate according to Chen et al., (2010).

2.3. Purification of extracellular proteins from P. piceum

The mycelia-free cultured medium was collected by centrifugation, and concentrated by ultrafiltration with a Biomax Mr. 10,000 cut-off Millipore membrane (Millipore, USA) (Gao et al., 2012). The concentrated culture filtrate was loaded onto the gel filtration chromatography (Sephadex S-200) (GE, Sweden) using AKTA purifier (GE, Sweden). The column was equilibrated and washed with 20 mM sodium acetate buffer, pH = 4.8 at a flow rate of 0.3 mL/min. Elution of the protein was monitored at 280 nm.

2.4. Determination of protein concentration

Protein concentrations were determined using a trace protein concentration determination kit via the bicinchoninic acid method (TransGen Biotech).

2.5. Substrate specificity

The substrate specificity of the purified protein from *P. piceum* was determined using different cellulosic substrate of 1% (w/v) carboxymethyl-cellulose, avicel, xylan, salicin. The reaction was carried out at 50 mM sodium acetate buffer, pH 4.8, 50 °C for 30 min. The total amount of reducing sugars in the supernatant was determined by the dinitrosalicylic acid (DNS) method (Gao et al., 2012).

2.6. Internal amino acid of purified protein by MALDI-TOF

Purified protein was identified via tandem MS (MS/MS) by Tianjin Biochip Corporation (Tianjin, China).

2.7. Biochemical characterization of purified protein

2.7.1. Effect of temperature and pH on β-glucosidase activity

The optimum temperature for purified protein was measured in 50 mM sodium acetate buffer (pH 4.8) at various temperatures from 30 to 70 °C. The optimum pH for purified protein was measured in 50 mM sodium acetate buffer at pH 2.0–7.0.

2.7.2. Determination of K_m and K_{cat}

Enzyme kinetic parameters of purified protein were obtained by measuring the rate of hydrolysis of *p*NPG at various concentrations (0.1–1.0%) (w/v) at optimum temperature for 30 min in 50 mM sodium acetate buffer (pH 4.8). The enzymatic kinetic parameters, K_m and K_{cat} were determined from the Lineweaver–Burk plots using the enzyme kinetics program.

2.8. Semi-quantitative reverse transcriptase polymerase chain reaction (semi-quantitative RT-PCR)

cDNA was used as a template for amplifying a partial sequence of gene. The primers of *Cel3b* were TTGGCTGGATGGGTGAGA and ACGGGTCCGAGAAGAACA, generating a segment of 225 bp DNA fragment. β-Actin was used as internal control to verify the RT-PCR reaction. The primers of β-actin were CTCCATCCAGGCCGTTCTG and CATGAGGTAGTCGGTCAAGTCAC, generating 150 bp DNA fragment. The transcription level of *PpCel3b* grown on induction condition and repression condition were quantitatively measured using the ImageJ software.

2.9. Transglycosylation assay towards glucose

To test the capacity of the purified protein to synthesize disaccharide derivatives, it was incubated with a highly concentrated solution (40 g/L glucose) at 50 °C for 72 h. The samples were separated on Aglient Zorbax Carbohydrate against standard markers (cellobiose, sophorose and gentiobiose) at 30 °C, using acetonitrile as eluent at a flow rate of 1.0 mL/min.

2.10. Synergism between the purified proteins and different enzyme samples

Supplementation experiments were performed in duplicate at 10-mL centrifuge tube. The reaction mixtures contained 5% (w/v) pretreated corn stover (water-washed) and 20 FPU of different commercial enzyme preparations per gram substrate in a total volume of 5-mL (cellulase from *T. reesei*; cellulase from *P. piceum*) with the supplementation dosage of 50 μg purified protein/g substrate. Saccharification was performed at 50 °C for 96 h. Glucose concentration was determined by SBA-4C biological sensor analyzer (Biological Institute of Shandong Academy of Science, China) (Gao et al., 2012).

3. Results and discussion

3.1. Purification of the extracellular protein from P. piceum

The concentrated culture filtrate of *P. piceum* was subjected to a gel filtration chromatography (Sephadex S200) (GE, Sweden) and yield two main peaks (Supplementation data Fig. 1). According to the principle of column chromatography, the molecular weight of the peak I was the highest. Surprisingly the protein was detected by SDS–PAGE as a single band (Fig. 1A). The protein had a molecular weight of 92 kDa (Fig. 1A). This protein was purified 8.7-fold with a specific activity of 80 IU/mg against *p*NPG as substrate (Supplementation data Table 1).

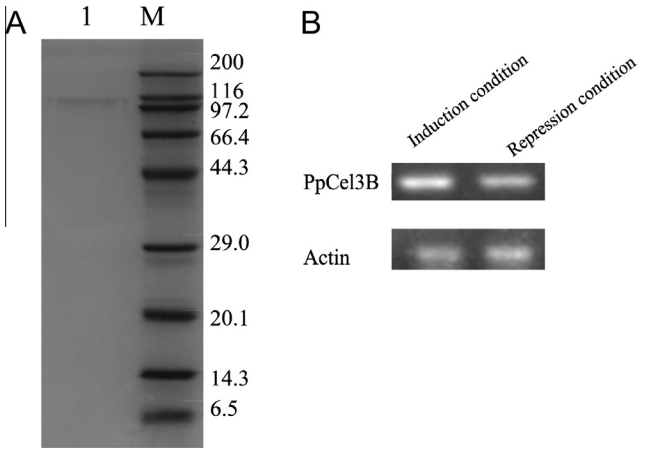

Fig. 1. (A) SDS–PAGE analysis of purified PpCel3B from *P. piceum*. Lane 1, purified PpCel3B of *P. piceum*. Lane M, protein marker (TransGen Biotech). (B) Semi-quantitative RT-PCR analysis of *cel3b* transcript in *P. piceum* grown on glucose and cellulose. β-Actin was used as internal control to verify the RT-PCR reaction.

3.2. Identification of the protein purified from P. piceum by MALDI-TOF (MS/MS)

A peptide with sequence of HYIANEQEHFR was found. The sequence of the protein was blasted using the search protocol blast. The peptide showed 99% similar to cel3b-like protein from *Beauveria bassiana*. The results of MALDI-TOF showed that the protein may be ranged to Cel3B belonging to family 3. The protein was designated as PpCel3B.

3.3. Substrate specificity

Cel3B, like Cel3A, had high activity of 23.4 IU/mg and 80 IU/mg against cellobiose and *p*NPG, respectively (Supplementation data Table 2). Moreover, PpCel3B was active on other β-1, 4 oligosaccharides with chain lengths of up to seven glucose units (data not shown). PpCel3B showed the highest activity against salicin with a specific activity of 408 IU/mg. It showed no activity for *p*NPC, CMC-Na, xylan and avicel. Surprisingly, PpCel3B had new enzymatic activity of 188 IU/mg against xylotiose. PpCel3B had a novel bifunctional activity like β-glycoside from *Chrysosporium lucknowense*, which was found to be a novel bifunctional glycoside hydrolase with both β-glucosidase and β-xylosidase activity (Dostenko et al., 2012).

3.4. Biochemical characterization of the purified β-glucosidase

The optimum temperature of the PpCel3B was observed at 60 °C and the enzyme activity was maintained 65% at 50 °C. The optimum pH of the PpCel3B was detected at pH 5.0, with 90% and 80% of the maximum activity appearing at pH4.0 and 6.0, respectively.

PpCel3B had an apparent K_m value of 0.003 mM, a V_{max} value of 3.1 mM/min, a K_{cat} value of 2.8 s^{-1}. The K_m for *p*NPG is much lower than that K_m values obtained for the β-glucosidase of *Aspergillus species* (0.2 mM to 1.6 mM), Bgl1D (0.54 mM) and Bgl1E (2.11 mM) from uncultured soil microorganisms (Jiang et al., 2011). The low K_m for the substrate is important in an industrial saccharification for reducing cellobiose inhibition exerted by other enzymes in cellulase systems.

3.5. Analyzing the biological function of PpCel3B

3.5.1. The role of PpCel3B in forming the soluble cellulose inducer compounds

HPLC results showed that PpCel3B successfully synthesized two important distinct disaccharide derivatives. The identification of the transglycosylation products were sophorose and gentiobiose. PpCel3B showed high tranglycosylation activity of 150 mg sophorose/mg protein and 75 mg gentiobiose/mg protein (Supplementation data Table 3). Several studies about tranglycosylation of Cel3A have been reported. *Aspergillus oryzae* Cel3A in was able to convert glucose to produced 52.48 mg/mL gentiobiose (He et al., 2013). *Fusarium oxysporum* Cel3A synthesizes cellotriose with 15–20% recovery and with initial substrate concentration of 16% cellulose and 40% gentibiose mixture (Christakopoulos et al., 1994). This paper showed that PpCel3B may be the β-glucosidase in the extracellular cellulase for inducer formation besides Cel3A. The β-glucosidase from zygomycete *Rhizomucor miehei* showed both transglycosylation and transgalactosylation activities resulting in formation of glycosides from cellobiose, lactose and ethanol (Krisch et al., 2012).

Besides the inducers by tranglycosylation, PpCel3B still could form other inducers. PpCel3B had novel enzymatic activity of 188 IU/mg against xylotriose to produce xylobiose and D-xylose (Supplementation data Table 2). Xylobiose and D-xylose could activate the regulatory transcription factor, which could improve the cellulase and hemicellulase production.

Semi-quantitative PCR analysis showed that *PpCel3b* could transcript both under repression and induction condition (Fig. 1B). The transcription level of *PpCel3b* was 2.2 times as much as that produced under repression condition (Supplementation data Fig. 2). It implied that *PpCel3b* was constitutive gene. In the initial phase of cellulase production by fungi, a low basal level of constitutive cellulase and hemicellulase were formed, which could start the degradation of cellulose and release small amounts of oligosaccharides and xylooligomer. PpCel3B could hydrolyze the oligosaccharides and xylooligomer, simultaneously and form multiple inducers for cellulase and hemicellulase. PpCel3B played an important role in production signaling molecules and boosting cellulase and hemicellulase synthesis.

3.5.2. The role of PpCel3B in boosting enzymatic degradation of delignified corn stover

In order to clarify the role of PpCel3B in boosting the enzymatic degradation of delignified corn stover, PpCel3B was supplemented, respectively, to different cellulases with a small dosage of 52 μg/g substrate in total of 5 mL volume. PpCel3B showed apparent synergism with cellulase from *T. reesei* and *P. piceum*. In the supplementation experiment, although the dosage of PpCel3B was small, the glucose yield released from delignified corn stover by the cellulase from *T. reesei* and *P. piceum* increased 15% and 35% respectively (Fig. 2).

Alkali pretreatment of corn stover resulted in a large number of hemicellulose existence. Qing et al. showed that xylanase activities in most commercial enzyme preparations have been shown to be insufficient to completely hydrolyze xylan, resulting in high xylooligomer concentrations remaining in the hydrolysis broth. The accumulation of xylooligomer resulted in the decrease of initial hydrolysis and a lower final glucose yield. A comparison among glucose sugars and xylose sugars also showed that xylooligomers were more powerful inhibitors than well-established glucose and cellobiose (Qing and Wyman, 2011). In the saccharification of cellulase from *T. reesei* and *P. piceum*, the main accumulation of xylooligomers was xylotriose (data not shown). PpCel3B showed a new enzymatic activity with 188 IU/mg against xylotriose. Due to the addition of PpCel3B, the accumulation of xylotriose apparently decreased (data not shown). Removing the effect of xylotriose and cellobiose inhibition by applying PpCel3B could play an important role in improving the lignocelluloses conversion.

The yields of glucose released by *T. reesei* cellulase 19.4% by addition of β-glucosidase (Novozyme 188) with 1.45 mg protein/g glucan (Zhang et al., 2010). The supplementation of commercial

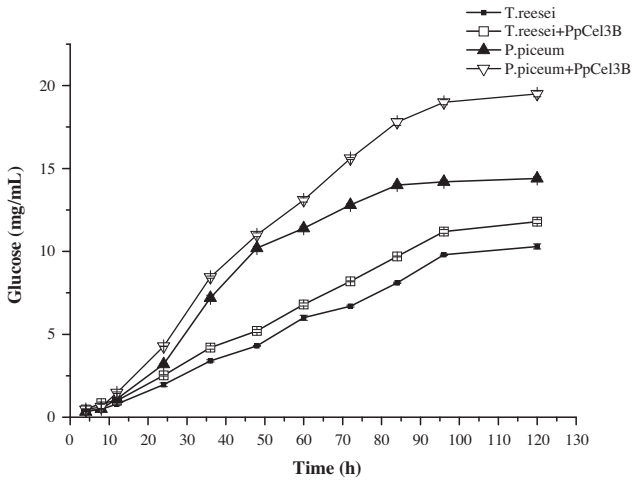

Fig. 2. Synergism between PpCel3B and different cellulase, with a supplementation dosage of 52 μg PpCel3B/g substrate.

β-glucosidase (60 IU/mL) to cellulase from *Penicillium* could improve biomass hydrolysis efficiency by 25.7% (Rajasree et al., 2013). The saccharifying ability of QM9414 cellulase toward corncob improved 76.4% by adding purified β-glucosidase (Novozyme NS-50010) at the ratio of FPA and β-glucosidase to 1:1. However, the promoting effect of PpCel3B was not as remarkable as mentioned by Chen et al., (2010), which could be due to the variations of β-glucosidase supplementation quantity and the different source of enzyme complex. Previous studies only demostrated commercial β-glucosidase play an important role in removing cellobiose inhibition. In this paper, the addition of PpCel3B to different cellulase preparation could help alleviate both effect of inhibition by cellobiose and xylooligomers, and thus facilitate cellulose saccharification.

4. Conclusion

Low activity and high cost of cellulase were the bottleneck for cellulase industrial production. PpCel3B played an important role in forming multiple soluble cellulose inducers via high transglycosylation activity and novel enzymatic activity. PpCel3B will be overexpressed in cellulytic fungi, which could boost cellulase and hemicellulase yield and simultaneously shorten its fermentation cycle. PpCel3B showed the strong synergism with different cellulases by removing multiple inhibitors for cellulase. Development of better enzyme cocktails using new enzyme component will stimulate cellulase efficiency.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2013.08.089.

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